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Cellular proteins mediate 5′–3′ end contacts of Norwalk virus genomic RNA

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ABSTRACT

Long-range RNA–RNA interactions between the 5′ and 3′ ends are a common feature involved in the regulation of both the initiation of translation and the synthesis of the viral genomic RNAs. These interactions either take place by direct RNA–RNA contacts or can be mediated by proteins. By *in silico* analysis, we found three possible complementary sequences (CS) between the 5′ and the 3′ ends of the Norwalk virus genomic RNA. Co-precipitation assays demonstrated that physical contacts between the 5′ and the 3′ ends of the NV genomic RNA were stabilized by cellular proteins. Mutations and deletions within these regions, that altered the formation of the CS-1 motif disrupted the 5′–3′ end contacts, while mutations that restore complementarity of the CS-1 motif, recover the ability to form these contacts. These results suggest that the NV genomic 5′–3′ end contacts initially occur by RNA–RNA interactions but are further stabilized by cellular proteins.

Introduction

Noroviruses (NoV) are the causative agents of viral gastroenteritis outbreaks worldwide (Lopman et al., 2008; Patel et al., 2008; Teunis et al., 2008) in all age groups and also cause sporadic cases of gastroenteritis (Arness et al., 2000; Fankhauser et al., 2002). These viruses are non-enveloped and have a 7.6-kb positive-sense single-stranded RNA genome, which contains three primary open reading frames (ORFs). ORF1 encodes the non-structural polyprotein, while ORF2 and ORF3 encode the major (VP1) and minor (VP2) structural proteins, respectively, which are translated from a subgenomic RNA molecule (Clarke and Lambden, 1997). The 5′-end sequence of the genomic and subgenomic RNAs are highly conserved among several members of the *Caliciviridae* family (Hardy and Estes, 1996), suggesting that this region might be important for viral replication. The genome of the Norwalk virus (NV), the prototype strain of human NoV, contains a 4 nt 5′ UTR and lacks a cap structure (Clarke and Lambden, 2000); and it is believed that it is covalently linked to a protein designated VPg (virion protein linked to genome) (Guix et al., 2007), which has been implicated in viral translation (Daughenbaugh et al., 2003, 2006; Goodfellow et al., 2005). The 3′ UTR is 66 nt long and is polyadenylated. As a positive polarity virus, it is expected that the genomic RNA has a dual function: first, it directs the synthesis of the non-structural viral proteins, and then, it acts as a template for the generation of negative polarity RNA molecules that serve as templates for the generation of progeny genomic positive polarity RNA molecules. The molecular features of the regulation of RNA translation and replication are still poorly understood; however, studies in other viruses suggest that both processes are mutually exclusive (Barton et al., 1999; Isken et al., 2006) and depend on regulatory elements that are primarily located in the 5′ and 3′ ends of the viral RNA genome (Martinez-Salas et al., 2008; Song et al., 2008). A study of the regions located at each end of the viral genomic RNA demonstrated their important role during the control of viral RNA translation and replication. The regulation of both processes must be strikingly modulated to permit efficient viral proliferation.

There is increasing, well documented evidence that long-distance interactions between both terminal ends of the genomic RNA molecule are a very common feature among positive-sense RNA viruses and support the coordination of viral protein and RNA synthesis (Alvarez et al., 2005; Filomatori et al., 2006; Gamarnik and Andino, 1998; Herold and Andino, 2001; Hu et al., 2007; Isken et al., 2003, 2007; Khromykh et al., 2001). These interactions can also occur in the anti-genomic RNAs (Huang and Lai, 2001). In this regard, each virus or group of viruses has developed its own strategies to allow these 5′–3′ end contacts depending on the sequences and or elements present within these regions. Some of these interactions can occur via RNA–RNA contacts, as in dengue virus genomic RNA, where sequence complementarity is required for RNA replication and viral viability (Alvarez et al., 2005, 2008; Filomatori et al., 2006). When sequence complementarity is not sufficient to direct these 5′–3′ end contacts, however, cellular proteins may act as facilitators, as is the case for bovine viral diarrhea virus (BVDV) and hepatitis C virus (HCV), where weak 5′–3′ end contacts are stabilized by the formation of a ribonucleoprotein complex with the nuclear factor associated with ds-RNA (NFAR) group of proteins (Isken et al., 2003, 2007). In the case of poliovirus (PV), the cellular PCBP and the viral 3CD proteins are bound

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to a cloverleaf structure present in the 5′ end of the genomic RNA and interact with the PABP bound to the poly A tail to promote the synthesis of the negative polarity RNA (Herold and Andino, 2001). On the other hand, for the foot-and-mouth disease virus (FMDV), the 5′–3′ end bridging involves two essential unrelated elements within the 5′ UTR, which promotes direct RNA–RNA contacts, and RNA–protein interactions. These bridges have been suggested to be involved in the switch from translation to replication (Serrano et al., 2006). For the mouse hepatitis virus (MHV), 5′–3′ end contacts occur due to an interaction between the PTB protein, which is bound to the 5′ end, and hnRNP A1, which is bound to the 3′ end. Both ends of the negative polarity RNA also interact via PTB–hnRNP A1 interactions, and it has been proposed that these contacts are important for RNA replication and transcription (Huang and Lai, 2001).

Since increasing evidence indicates that long-range RNA interactions within the viral genomic or negative polarity RNA are a very common viral replicative strategy, it is likely that these types of interactions also take place in members of the *Caliciviridae* family. In this regard, a bioinformatic and functional analysis of RNA second structure elements among these viruses demonstrated that conserved stem-loop structures within the 5′ and 3′ ends of the genome as well as in the subgenomic (sg) promoter had important roles during viral infectivity (Simmonds et al., 2008). Therefore, specific interactions between the 5′ and 3′ ends of these RNAs, and high-order RNA structures at the termini of viral genome RNA, might take place for the regulation of translation and viral RNA synthesis. The role of proteins in mediating 5′–3′ end contacts to coordinate and promote viral translation and RNA replication is well documented (De Nova-Ocampo et al., 2002; Gamarnik and Andino, 1998; Huang and Lai, 2001; Isken et al., 2003, 2007). In particular for members of the calicivirus family, several cellular proteins have been reported to bind to the 5′ end of the NV genomic RNA (Gutierrez-Escolano et al., 2000, 2003) and to participate in different steps of viral replication (Karakasiliotis et al., 2006; Chaudhry et al., 2006; Daughenbaugh et al., 2003, 2006; Goodfellow et al., 2005). Therefore, calicivirus genomes possess *cis* and *trans* elements that could direct interactions between the 5′ and 3′ ends.

In this report, we provide *in vitro* experimental evidence indicating that the 5′ and 3′ ends of the genomic RNA of NV interact in the presence of cellular proteins. Moreover, the integrity of a 6 nucleotide (nts) complementary sequence (CS-1) predicted *in silico* is required to maintain these *in vitro* RNA–RNA contacts in the presence of cellular proteins.

Results

RNA–RNA contacts

To determine if RNA sequences present in the 5′ end interacted with the 3′ UTR of the NV genomic RNA, *in silico* assays were carried out using the Mfold-2 program (Zuker, 2003). The RNA sequences analyzed consisted of the first 158 nts of the 5′ end and the polyadenylated 3′ UTR of the NV genome. The size of this RNA was chosen because it contains the majority of the most conserved predicted stem-loop elements in the 5′ end (Jiang et al., 1993; Simmonds et al., 2008), and is upstream of an in frame AUG codon. A poly N sequence was used as a spacer region between both ends. Two alternative folding predictions with similar energies were obtained. In structure I ($\Delta G = -64.7$), a base pairing region formed from a palindromic sequence present in the first 60–65 nts of the 5′ end (ACAACA) and nts 7584–7589 within the 3′ UTR (UGUUGU) was predicted (Fig. 1A). This complementary sequence was named CS-3 (Fig. 1A). In structure II ($\Delta G = -64.4$), (Fig. 1B), two additional base pairing regions corresponding to nts 5–10 and 32–36 of the 5′ end and nts 7605–7615 within the 3′ UTR were predicted. These regions were named the CS-1 and CS-2 motifs, respectively (Fig. 1B).

In an attempt to determine if the interactions between the 5′ and 3′ ends of the NV genomic RNA predicted in the *in silico* assays also occurred *in vitro*, co-precipitation assays were performed with the NV [α-^{32}P]-labeled polyadenylated 3′ UTR RNA and the biotin-labeled 5′ RNA. This assay allowed the detection of 5′–3′ complex formation only when the labeled RNA was co-precipitated with streptavidin beads, due to their interaction with the biotin-labeled RNA (see Materials and methods). Under the conditions used in this assay, an interaction between the 5′ and 3′ ends of the NV genomic RNA was not detected (Fig. 2, lane 2).

Cellular proteins mediate the interaction between the 5′ and 3′ ends of the NV genomic RNA

Since RNA–RNA interactions between the terminal regions of the NV genomic RNA alone were not detected, we evaluated the requirement for cellular proteins to mediate these interactions. Previous results from our laboratory have demonstrated that both the 5′ and 3′ terminal regions of the NV genomic RNA used in the RNA–RNA interaction assay bind to several cellular proteins such as La, PTB, PCBP-2, and PABP (Gutierrez-Escolano et al., 2000). The ability of both ends of the NV genomic RNA to interact with cellular proteins that promote 5′–3′ end contacts in other viral genomes suggests that such proteins could also promote an interaction between both ends of the NV genomic RNA. To explore this possibility, co-precipitation assays were performed with an [α-^{32}P]-labeled polyadenylated 3′ UTR RNA and a biotin-labeled 5′ RNA incubated in the presence of cellular proteins. Therefore, both the [α-^{32}P]-labeled 3′ UTR and the biotin-labeled 5′ RNAs were incubated in the absence or presence of cellular proteins from HeLa and CaCo-2 cells. Only in the presence of cellular proteins from HeLa (Fig. 2, lane 5) and CaCo-2 cells (Fig. 2, lane 6) was the [α-^{32}P]-labeled polyadenylated 3′ UTR RNA co-precipitated with the biotin-labeled 5′ RNA, indicating that the interaction between both ends only occurred in the presence of cellular proteins. Moreover, these interactions were specific since no [α-^{32}P]-labeled signal was detected in the presence of a non related protein such as BSA (Fig. 2, lane 3) or a non related biotin-labeled RNA (Fig. 2, lane 4).

The first nts of the 5′ terminal region of the NV genomic RNA are necessary for the interaction with the 3′ UTR

Since the 5′–3′ end contacts of the NV genome only take place in the presence of cellular proteins, the possibilities are: 1) that the proteins bound to each 5′ and 3′ end mediate the interactions, or 2) that weak RNA–RNA contacts are stabilized by cellular proteins. To distinguish between these two possibilities and to analyze the influence of the complementary regions that could participate in these 5′–3′ end contacts, we induced disruptions of the base paired regions (CS-1, CS-2 and CS-3).

The CS-1 motif is located within the first nts of the genome, which is a highly conserved region in the genomic and subgenomic RNAs and contains regulatory elements such as the three conserved tandem AUG codons where the translation initiation takes place. For this reason, four mutations that disrupted the three tandem AUG codons located within the first 13 nts (AUCAUCAAC) and a deletion of the first 13 nts of the 5′ end were used in two RNA molecules named 5′ mutated and 5′ deleted regions, respectively. *In silico* interaction assays with the 5′ mutated and 5′ deleted regions predicted that the CS-1 and CS-2 motifs were not formed, while the CS-3 motif remained unaltered. The predicted folding structures have energies of $\Delta G = -62.7$ for the 5′ mutated and $\Delta G = -60.0$ for the 5′ deleted regions.

The co-precipitation assays performed with both the 5′ mutated and 5′ deleted regions showed that a complex formation was only detected in the presence of the biotin-labeled 5′ wt 1–158 and the [α-^{32}P]-labeled polyadenylated 3′ UTR, (Fig. 3B, lane 5). With the biotin-labeled 5′ mutated and 5′ deleted RNAs, a significant reduction

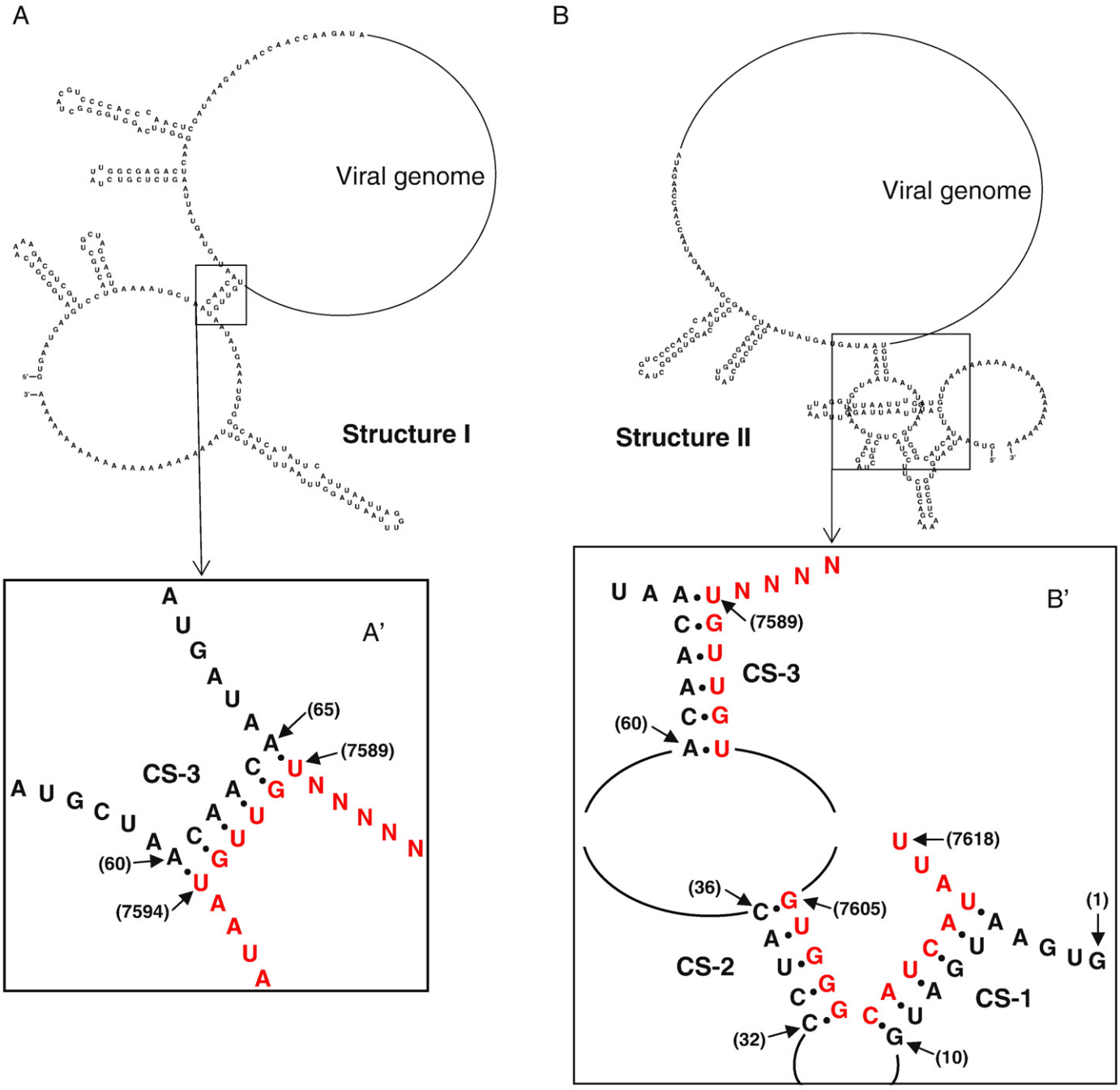

Fig. 1. Computer analysis of the 5′–3′ end contacts of the NV genomic RNA wt region. (A and B) Two predicted secondary structures (I and II) show complementary sequences (CS) formed between the first 158 nt and the last 66 nt (3′ UTR) plus a poly (A) tail of the NV genomic RNA. The viral genome is represented schematically with a solid line linking both terminal regions of the molecule. The predicted secondary structures I and II were those with the lowest ΔG (−64.7 and −64.4 kcal mol^{-1}, respectively). Both predictions were performed using Mfold 2 software http://mfold2.wustle.edu). (A′ and B′) Expanded structure showing complementary regions (CS-1, CS-2 and CS-3) formed in the predicted structures I and II, respectively. Positions of nucleotide numbers in the genome are indicated.

in the co-precipitation of the [α-^{32}P]-labeled polyadenylated 3′ UTR RNA was observed (Fig. 3B, lanes 7 and 6 respectively). These results indicate that the integrity of the first 13 nts from the 5′ terminal end of the NV genomic RNA, which form the CS-1 motif, is important for the interaction with the polyadenylated 3′ UTR in the presence of cellular proteins. Since the CS-2 motif was also disrupted, its formation could also influence the 5′–3′ end contacts.

To further confirm that the 5′–3′ end contacts are important for the complex formation, compensatory mutations, in the polyadenylated 3′ UTR RNA that restore complementarity with the 5′ mutated RNA, were included in an RNA molecule named 3′ comp (A) region (Fig. 3A). *In silico* interaction assays with the 5′ mutated and 3′ comp (A) regions showed that the interaction was restored in the CS-1 structure. This structure has an energy of $\Delta G = -64.20$. The co-precipitation assays performed with the [α-^{32}P]-labeled 3′ comp (A) and a biotin-labeled 5′ mutated RNA, showed that, the [α-^{32}P]-labeled 3′ comp (A) was co-precipitated in the presence of cellular proteins (Fig. 3B, lane 8), indicating that the 5′–3′ end contacts are important for the complex formation. However, the [α-^{32}P]-labeled signal precipitated was weaker than the one obtained when the [α-^{32}P]-labeled polyadenylated 3′ UTR was co-precipitated with the 5′ wt 1–158 RNA (Fig. 3B, lanes 5 and 8 respectively). These results support the notion that a

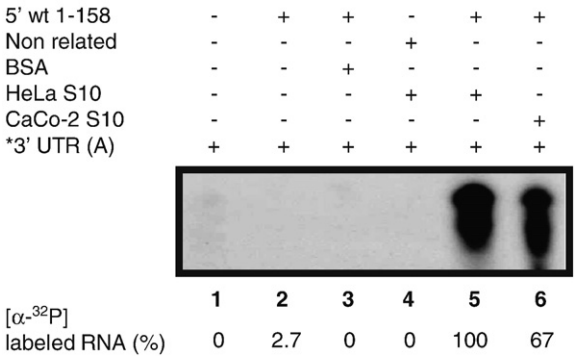

Fig. 2. Co-precipitation assay of the 5′ and 3′ ends of the NV genomic RNA in the presence of cellular proteins. Co-precipitation assay of the [α-^{32}P]-labeled polyadenylated 3′ end (lanes 1–6) and the biotin-labeled 5′ wt 1–158 (lanes 2, 3, 5 and 6) of the NV genomic RNA or a non related biotin-labeled RNA (lane 4) in the absence (lanes 1 and 2) or presence of 10 μg BSA, (lane 3), 10 μg HeLa S10 (lanes 4 and 5) or 10 μg CaCo-2 S10 extracts (lane 6). Quantification of the precipitated [α-^{32}P]-labeled RNA fraction is indicated in the bottom of the gel and expressed as percentage of intensity obtained with wt region. Data shown are representative of several independent experiments.

complementary region is required for the 5′–3′ end contacts in the presence of cellular proteins.

The first 20 nts of the NV genomic RNA are sufficient for the interaction with the polyadenylated 3′ UTR

Since modifications in the first nts of the NV genome, which contain the three AUG codons, altered the complementary sequences of the CS-1 motif and reduced the ability of the 5′ end to bind to the polyadenylated 3′ UTR, we analyzed the ability of the first 20 nts of the NV genome to support the 5′–3′ contacts. The first 20 nts contain the unaltered AUGAUGAUG sequence and can form the CS-1 motif but do not contain the nts that form the CS-2 motif. Therefore, we carried out co-precipitation assays with the [α-^{32}P]-labeled polyadenylated 3′ UTR RNA and a biotin-labeled RNA containing the first 20 nts of the NV genome, named 5′ wt 1–20, in the presence or the absence of HeLa cell extracts. Under these conditions, the [α-^{32}P]-labeled 5′ wt 1–20 RNA was co-precipitated with the biotin-labeled polyadenylated 3′ UTR in the presence of cellular proteins (Fig. 4A, lane 5); however, no labeled co-precipitated signal was detected when the assays were performed without cellular proteins (Fig. 4A, lane 2) or in the presence of a non related RNA (Fig. 4A, lane 4) or protein (Fig. 4A, lane 3). These results indicate that the first 20 nts of the NV genome containing the AUGAUGAUG nts and CS-1 motif are sufficient to promote the interaction between the 5′ and 3′ ends.

If the first 20 nts are sufficient to support the interaction with the polyadenylated 3′ UTR, mutations within the first 13 nts, that contain the AUCAUCAAC sequence, should also prevent the 5′–3′ end contacts in the context of this shorter RNA. Therefore, we carried out co-precipitation assays with a [α-^{32}P]-labeled polyadenylated 3′ UTR and the biotin-labeled 5′ mut 1–20 RNAs in the presence or absence of HeLa cell extracts. A significant reduction in the co-precipitation of the [α-^{32}P]-labeled polyadenylated 3′ UTR compared to wt region RNA was observed (Fig. 4A, lane 6). This result indicates that the integrity of the AUGAUGAUG sequence within the 13 nts from the 5′ terminal end of the NV genomic RNA, in the context of both a 20 and a 158 nts long RNA, is necessary for the interaction with the polyadenylated 3′ UTR in the presence of cellular proteins (Fig. 4A, lane 6 and Fig. 3B, lane 7 respectively). To further determine if mutations in the 3′ comp (A) RNA, that restore the 5′–3′ end contacts with the biotin-labeled 5′ mutated RNA, could also restore the interactions with the 5′ mut 1–20 RNA, co-precipitation assays were performed. The [α-^{32}P]-labeled 3′ comp (A) was able to be co-precipitated with the biotin-labeled 5′ mut 1–20 RNA (Fig 4A, lane 7), indicating that the formation of a complementary region is important for the 5′–3′ end contacts in the presence of cellular proteins, as shown with the 5′ mutated RNA (Fig 3A, lane 8). Although 3′ comp (A) RNA was able to compensate the mutations present in the 5′ mut RNA, the amount of co-precipitated [α-^{32}P]-labeled RNA precipitated was lower when compared with the 5′ wt 1–20 RNAs (Fig. 4A, lanes 7 and 5 respectively). These results support our previous conclusion that the presence of complementary sequences within the 5′ and the 3′ ends is important for the complex formation in the presence of cellular proteins.

Since the first 20 nts of the 5′ end were sufficient for interactions with the polyadenylated 3′ UTR, and this interaction was dependent on cellular proteins, we next tried to determine which proteins bound to the 5′ 1–20 RNA. A UV-crosslinking assay with both the [α-^{32}P]-labeled 5′ wt 1–20 and the non related RNAs and HeLa cell extracts was performed. The UV-crosslinking pattern of the [α-^{32}P]-labeled 5′ wt 1–20 RNA revealed that eight proteins with molecular weights of 110, 97, 75, 68, 60/57, 52, 50 and 42 kDa bound to the first 20 nts (Fig. 4B, lane 2). This protein pattern was almost identical to the one seen with the proteins that bound to the 5′ 1–158 wt RNA (Gutierrez-

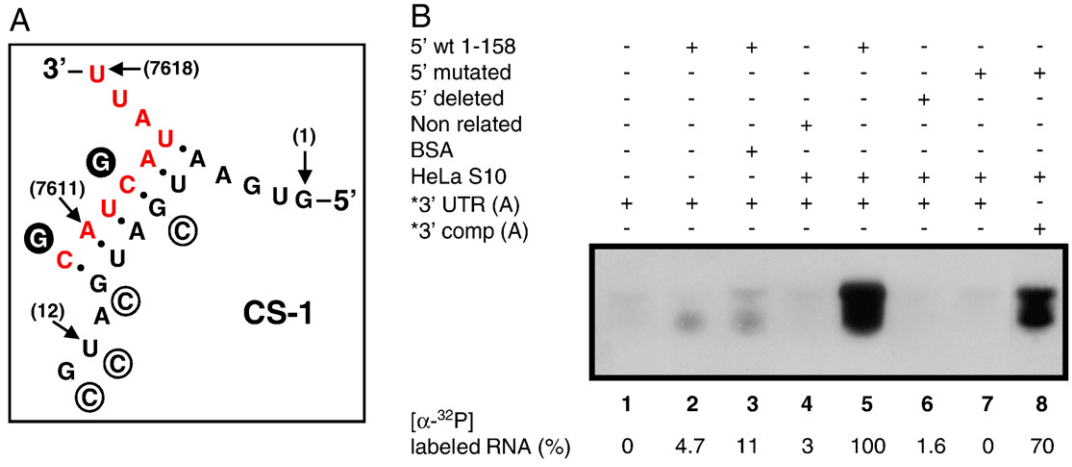

Fig. 3. Co-precipitation assays of the 5′ wt, 5′ mutant, 5′ deleted and 3′ compensatory regions of the NV genomic RNA. (A) Base pairing region of the CS-1 motif showing positions of introduced mutations in the 5′ end (unfilled circles) and compensatory mutations in the 3′ end (filled circles). (B) Co-precipitation assay of the [α-^{32}P]-labeled polyadenylated 3′ UTR (lanes 1–7) or the [α-^{32}P]-labeled polyadenylated 3′ compensatory (lane 8) RNAs and the biotin-labeled 5′ wt 1–158 (lanes 2, 3 and 5), 5′ mutated (lanes 7 and 8), 5′ deleted (lane 6) and non related (lane 4) RNAs in the absence (lanes 1 and 2) or presence of 10 μg BSA (lane 3) or 10 μg HeLa S10 (lanes 4–8). Quantification of the precipitated [α-^{32}P]-labeled RNA fraction is indicated in the bottom of the gel and expressed as percentage of intensity obtained with wt region. Data shown are representative of three independent experiments.

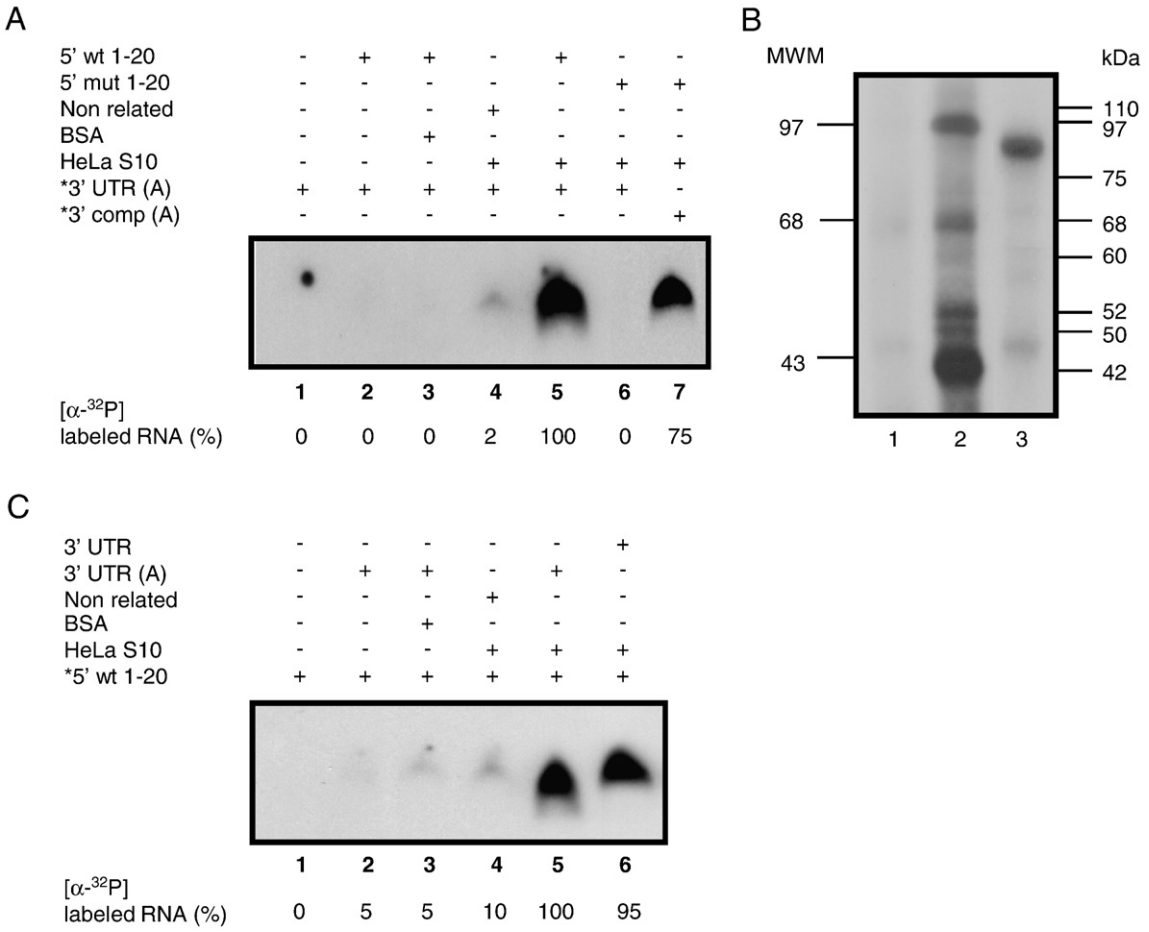

Fig. 4. Co-precipitation assays and UV-crosslinking pattern of the first 20 nts of the NV genomic RNA. (A) Co-precipitation assay of the [α-^{32}P]-labeled polyadenylated 3′ UTR (lanes 1–6) or the [α-^{32}P]-labeled polyadenylated 3′ compensatory (lane 7) RNAs and the biotin-labeled 5′ wt 1–20 (lanes 2, 3 and 5), 5′ mutated 1–20 (lanes 6 and 7), and non related (lane 4) RNAs in the absence (lanes 1and 2) or presence of 10 μg BSA (lane 3) or 10 μg HeLa S10 (lanes 4–7). Quantification of the precipitated [α-^{32}P]-labeled RNA fraction is indicated in the bottom of each gel and expressed as percentage of intensity obtained with wt region. Data shown are representative of three independent experiments. (B) UV-crosslinking pattern of the [α-^{32}P]-labeled first 20 nts of the NV genomic RNA (lanes 1 and 2) or non related RNA (lane 3) in the absence (lane 1) or presence (lanes 2 and 3) of S10 extracts from HeLa cells. The molecular weight of the UV-crosslinking proteins is shown on the right side of the figure. The molecular weight markers (MWM) are shown. (C) Co-precipitation assays with the [α-^{32}P]-labeled first 20 nts of the NV genomic RNA (lanes 1–6) and the biotin-labeled polyadenylated 3′ UTR (lanes 2, 3 and 5), 3′ UTR (lane 6), and non related RNAs (lane 4) in the absence (lanes 1 and 2) or presence of 10 μg BSA (lane 3) or 10 μg HeLa S10 extracts (lanes 4–6). Quantification of the precipitated [α-^{32}P]-labeled RNA fraction is indicated in the bottom of each gel and expressed as percentage of intensity obtained with wt region. Data shown are representative of three independent experiments.

Escolano et al., 2000). The non related RNA used in this assay, the human IRE-fer stem-loop element, was able to crosslink with two proteins of 90 and 45 kDa, as described previously, that do not correspond to any of the proteins crosslinked to the 5′ wt 1–20 RNA (Fig. 4B, lane 3) (Solano-González et al., 2007). The fact that the 5′ wt 1–20 RNA interacts with the 3′ UTR and forms the CS-1 motif, in addition to its ability to bind to almost all of the proteins that bind to the 5′ 1–158 wt RNA, supports the idea that this small region is sufficient to establish 5′–3′ end interactions.

Deletions in the 3′ UTR that alter the formation of the CS-1 motif affect the interaction with the first 20 nts of the NV genomic RNA

The 3′ UTR as well as the polyadenylated tail are important regulatory regions for several viruses (Bergamini et al., 2000; Borman et al., 2002). In particular, the polyadenylated NV 3′ UTR is 66 nts long and contains a stem-loop element that is conserved among members of the *Caliciviridae* family. The integrity of this element, at least in the genome of the murine norovirus (MNV), is important for viral viability (Simmonds et al., 2008).

To determine the elements within the polyadenylated 3′ UTR that could be involved in 5′–3′ end contacts, we used co-precipitation assays to analyze the role played by the polyadenylated 3′ UTR as well as the sequences involved in the *in silico* 5′–3′ interactions. When either the polyadenylated or non-polyadenylated [α-^{32}P]-labeled 3′ UTR was incubated with biotin-labeled 5′ wt 1–20 RNA in the presence of HeLa cell extracts, a similar amount of the co-precipitated [α-^{32}P]-labeled RNA was observed (Fig. 4C, lanes 5 and 6 respectively). This result indicates that the presence of the polyadenylated tail was not necessary to promote this specific 5′–3′ contacts between the 5′ wt 1–20 and the 3′ UTR RNAs.

Due to the fact that the complementary sequence promoting the formation of the CS-1 motif is located within the 3′ UTR, three RNAs that included deletions within this sequence were analyzed. 1) The 3′ 1–47 RNA contained the first 47 nts of the 3′ UTR (nts 7589–7634 of the NV genomic RNA) and included the complementary CAUCAU sequence present in the CS-1 motif and half of the conserved 3′ stem-loop structure. 2) The 3′ 22–66 RNA contained the sequences that form the stem-loop element (nts 7615 to 7654) and included the CAUCAU region. 3) The 3′ 1–21 RNA contained the first 21 nts of the 3′ UTR (nts 7589 to 7609 of the NV genomic RNA) and lacked the complementary CAUCAU sequence (Fig. 5A).

When co-precipitation assays were performed with the [α-^{32}P]-labeled 5′ 1–20 RNA containing the 5′ CS-1 AUGAUGAUG and the biotin-labeled 3′ UTR 3′ 1–47 and 3′ 22–66 RNAs containing the 3′ CS-1 CAUCAU, we detected the 5′–3′ end contacts (Fig. 5B, lanes 7

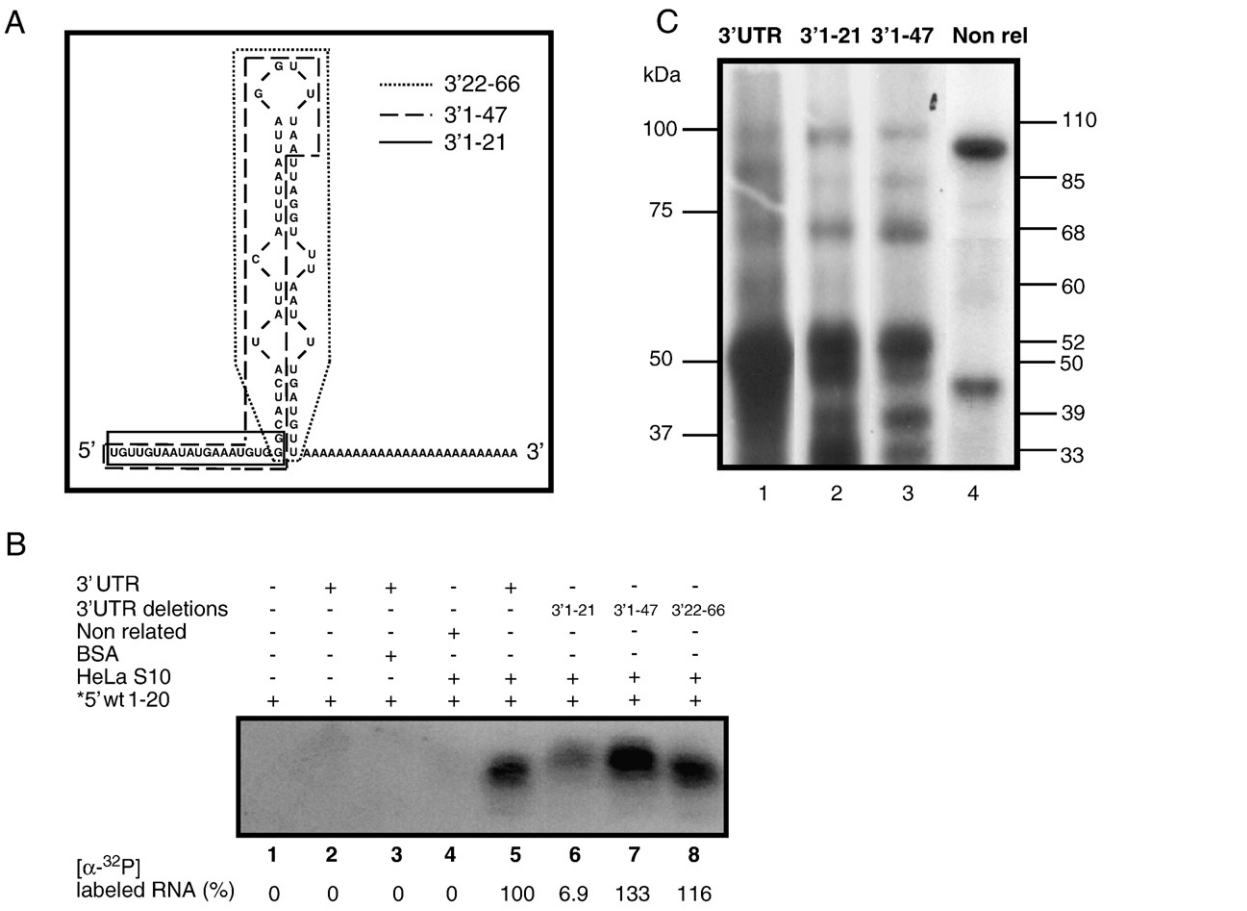

Fig. 5. Co-precipitation assays and UV-crosslinking patterns of the complete wt and deleted regions of the 3′ UTR of the NV genomic RNA. (A) Schematic representation of the complete and deleted polyadenylated 3′ UTRs. The deleted regions 3′ 1–21, 3′ 1–47 and 3′ 22–66 are indicated in the figure. (B) Co-precipitation assay of the [α-^{32}P]-labeled first 1–20 nts of the NV genomic RNA (lanes 1–7) and the complete biotin-labeled 3′ UTR (lanes 2, 3 and 5), deleted 3′ 1–21 (lane 6), deleted 3′ 1–47 (lane 7), deleted 3′ 22–66 (lane 8), and non related RNA (lane 4) in the absence (lanes 1–2) or presence of 10 μg BSA (lane 3), or 10 μg HeLa S10 extracts (lanes 5–8). Quantification of the precipitated [α-^{32}P]-labeled RNA fraction is indicated in the bottom of each gel and expressed as percentage of intensity obtained with wt region. Data shown are representative of three independent experiments. (C) UV-crosslinking patterns of the 3′ 1–21 and 3′ 1–47 RNAs. UV-crosslinking pattern of the [α-^{32}P]-labeled 3′ UTR (lane 1), the deleted 3′ 1–21 and deleted 3′ 1–47 RNAs containing the first 21 and 47 nts of the NV 3′ UTR (lanes 2 and 3 respectively) and the non related RNA (lane 4) in the presence of S10 extracts from HeLa cells. The molecular weights of the UV-crosslinking proteins are shown on the right side of the figure. The molecular weight markers (MWM) are shown.

and 8 respectively). On the other hand, when the 3′ 1–21 RNA lacking the 3′ CS-1 CAUCAU was tested, a significant reduction in the precipitation of the [α-^{32}P]-labeled 5′ 1–20 RNA was observed (Fig. 5B, lane 6). This result indicates that the presence of the 3′ CS-1 CAUCAU is essential for the formation of the 5′–3′ end complexes. These interactions were specific since no co-precipitation was observed when the assay was performed in the presence of non related RNA (Fig. 5B, lane 4) or protein (Fig. 5B, lane 3). Taken together, these results suggest that the complementary sequences that form the CS-1 motif are crucial for the formation of the 5′–3′ complexes of the NV genomic RNA.

Since the presence of the 5′ CS-1 and 3′ CS-1 is necessary for the formation of the 5′–3′ end complex, it is likely that RNA–RNA interactions are required for the formation of stable complexes mediated by cellular proteins. Since the CS-1 motif as well as cellular proteins were required for the 5′–3′ end interactions, we tested the ability of both the 3′ 1–21 RNA, which does not contain the complementary CAUCAU sequence and was not able to form the 5′–3′ end contacts, and the 3′ 1–47 RNA, which contains the CAUCAU complementary sequence and was able to form the 5′–3′ end contacts, to bind cellular proteins. The UV-crosslinking patterns obtained with both the [α-^{32}P] 3′ 1–21 and 3′ 1–47 RNAs were very similar to the UV-crosslinking pattern of the complete 3′ UTR (Fig. 5C, lanes 2, 3 and 1, respectively). The non related RNA used in this assay was able to crosslink with two proteins of 90 and 45 kDa that do not correspond to any of the proteins present in the UV-crosslinking patterns of the 3′ 1–21 and 3′ 1–47 (Fig. 5C, lanes 4, 2 and 3 respectively). The observation that both the 3′ 1–21 and the 3′ 1–47 RNAs showed similar protein binding patterns but differed in their ability to form 5′–3′ end contacts supports the notion that complexes are formed by initial RNA–RNA interactions and are subsequently stabilized by proteins.

Discussion

Long-range RNA–RNA interactions between the 5′ and 3′ ends are a very common feature involved in the regulation of both translation initiation and the synthesis of the viral genomic RNAs (Herold and Andino, 2001; Huang and Lai, 2001; Wells et al., 1998). Positive-sense RNA viruses have developed different strategies for 5′–3′-end interactions: while some occur by direct RNA–RNA contacts between complementary sequences, others are mediated by RNA binding proteins. In some cases, both kinds of interactions can take place within the same RNA (Serrano et al., 2006). In this study we provide direct evidence for a physical interaction between the 5′ and 3′ ends of the NV genomic RNA, which is sequence-mediated and further stabilized by cellular proteins.

Due to the fact that RNA–RNA contacts within several viral genomes were first determined using computational analysis, we

evaluated the presence of possible RNA–RNA contacts between the 5′ and 3′ ends of the NV genome RNA using the Mfold program (Zuker, 2003). Two alternative folding predictions were obtained with similar energies and contained a hexanucleotide palindromic complementary sequence formed between nts 60–65 of the 5′ end (5′-ACAACA) and nts 7589-7594 within the 3′ UTR (5′-UGUUGU), named CS-3 (Figs. 1A′ and B′). Structure II showed two additional base paired regions involving interaction between nts 5–10 and 32–36 of the 5′ end and nts 7605–7615 of the 3′ UTR (Fig. 1B′, CS-1 and CS-2 motifs, respectively). Nts 5–10 contain the sequence AUGAUG, which is located within a conserved region between the genomic and subgenomic RNAs and base pairs with the CAUCAU sequence of nts 7610–7615 within the 3′ UTR (Fig. 1B). Short motifs of 4–5 nts and 6–8 nts have also been reported to mediate functional long-distance interactions in other viruses (Hu et al., 2007; Khromykh et al., 2001; Serrano et al., 2006). Therefore, it is not surprising that this particular short complementary region in the NV genome could mediate contacts between the 5′ and 3′ ends of NV RNAs. It was also observed that the 5′–3′ end contacts between the first 20 nts from the 5′ end and 3′ UTR were independent of the polyadenylated tail, as occurs in other polyadenylated viral RNAs, however, we do not rule out the possibility that additional 5′–3′ interactions could depend on the presence of the polyadenylated tail (Huang and Lai, 2001; Serrano et al., 2006).

Our results with NV indicate that RNA–RNA interactions are promoted or stabilized by cellular proteins, as has been observed in other viruses. Two types of strategies have been documented for long-range RNA interactions supported by cellular proteins. One involves the proximity of the 5′ and 3′ ends promoted by the proteins bound to each region (Huang and Lai, 2001), and the other involves the presence of complementary sequences between both ends of the RNA that require cellular proteins to be stabilized (Isken et al., 2003). In the case of the 5′–3′ end contacts of the NV RNAs, both scenarios could promote the interactions.

In this regard, mutations and deletions within the 5′ and the 3′ ends that altered the formation of the CS-1 motif disrupted the 5′–3′ end contacts *in vitro*, even in the presence of cellular proteins, suggesting that the changes introduced into the 5′ and the 3′ ends, prevent protein binding rather that RNA–RNA interactions. However both the 3′ 1–21 RNA lacking the 3′ CS-1 and the 3′ 1–47 RNA containing the 3′ CS-1 bound to similar cellular proteins. On the other hand, mutations within the 3′ end that restore complementarity with the mutated 5′ end, permitted the 5′–3′ end contacts and the complex formation in the presence of cellular proteins. All these results indicate that specific complementarity between the 5′ CS-1, present in the 5′ end, and 3′ CS-1, present in the 3′ UTR of the NV genomic RNA is necessary to promote these contacts. The precipitated radiolabeled signals obtained with the restored CS1 motif, were weaker than the ones obtained with the wt CS1 motif, suggesting that, to some extent, changes in the sequences might affect the binding efficiency of the proteins. Taken together, these results indicate that NV genomic 5′–3′ end contacts occur initially by RNA–RNA interactions, then require further stabilization by cellular proteins.

Some proteins that promote or stabilize long-range RNA–RNA interactions have already been identified, including PCBP-2 and PABP for the PV genomic RNA (Herold and Andino, 2001), PTB and hnRNP A1 for the positive and negative polarity RNAs of the MHV (Huang and Lai, 2001), and members of the NF90/NFAR for the BVDV and HCV. Proteins La, PTB, PCBP-2, and PABP have also been reported to bind to the 5′ and 3′ ends of the NV and FCV genomic RNA (Gutierrez-Escolano et al., 2000, 2003; Karakasiliotis et al., 2006). It will be interesting, therefore, to determine if these proteins are actually present in the complexes that mediate the long-range contacts of the calicivirus genomic RNAs.

Even though the functional role of the 5′–3′ end contacts described in this work was not evaluated, it is possible that the interaction points that bring together the 5′ and 3′ ends of the NV genome could be a transitional conformation required to efficiently coordinate viral gene expression and RNA replication, something that is known to occur in other viruses (Alvarez et al., 2005; Herold and Andino, 2001; Isken et al., 2007; Khromykh et al., 2001). Still, the integrity of the complete stem-loop structures within the 3′ UTR – and probably in the 5′ end – seems to be required to promote a context that allows viral protein and RNA synthesis to take place. In this regard, the integrity of the corresponding stem-loop elements in the MNV is critical for its replication; thus, it may be crucial for NV replication as well (Simmonds et al., 2008).

Recent studies have shown that a phosphorodiamidiate morpholino oligomer (PMO) targeted against the first 19 nts of the conserved 5′ end of human NoV and the homologous region of the MNV genomes inhibits protein expression. The authors hypothesized that besides a reduction in MNV infectivity due to translation inhibition, other mechanisms such as interference with the RNA secondary structure essential for viral RNA replication could take place (Bok et al., 2008). In agreement with this hypothesis, the PMOs directed against the 5′ end of the genome could also inhibit the formation of the CS-1 motif, thereby affecting in some way viral replication. Comparative studies between *in vitro* and *in vivo* systems are necessary, however, to fully understand the role of these interactions in the NV replicative cycle. Finally, the role of the VPg protein, which is covalently linked to the 5′ end of the NV RNA, should be evaluated, although it has been proposed in other viral systems that 5′–3′ end contacts could take place without the participation of this protein (Gamarnik and Andino, 1998; Herold and Andino, 2001; Dreher and Miller, 2006).

Materials and methods

Cell cultures

HeLa cells were grown in Dulbecco's minimal essential medium supplemented with 10% newborn calf serum, 5000 U of penicillin, and 5 μg/ml of streptomycin in a 5% CO_2 incubator at 37 °C. The culture medium was changed every other day until the cells reached confluence. CaCo-2 cells were grown in Dulbecco's minimal essential medium containing 0.11% glutamine, 0.02% sodium pyruvate, 0.47% NaCl, 13 nonessential amino acids, 5000 U of penicillin, 5 μg/ml of streptomycin, and 10% fetal bovine serum. Both cell lines were grown in a 5% CO_2 incubator at 37 °C.

Computer-generated secondary structures of the 5′ and 3′ end contacts of NV genomic RNA

Computer analysis of the first 20 and 158 nts of the 5′ end and the 3′ UTR of the NV genomic RNA were performed using Mfold2 software (Zuker, 2003) through the web interface at http://frontend.bioinfo.rpi.edu/applications/mfold/.

In vitro transcription of the RNA molecules that contain different regions of the NV genomic RNAs

Two RNA molecules corresponding to the first 20 and 158 nt from the NV 5′ end and the complete 3′ UTR with or without the poly (A) tail and partial sequences 3′ 1–21 (nt 3′ 7589–7609); 3′ 1–41(nt 7589–7637) and 3′ 22–66 (7609–7654) of the NV genomic RNA were synthesized by *in vitro* transcription using T7 RNA polymerase from a template of PCR-amplified cDNAs containing the respective regions (Gutierrez-Escolano et al., 2003). The PCR was performed using the complete NV cDNA as a template, which was generously donated by X. Jiang (Cincinnati Children's Hospital Medical Center, Ohio). All sense primers used in the PCR contained the bacteriophage T7 promoter sequence. The PCR reactions for the 5′ amplicons were performed at 94 °C for 1 min, the corresponding annealing temperature for each

amplicon for 1 min (5′ 158, at 56 °C; 5′ mutated region, at 56 °C; 5′ deleted region, at 46 °C; 5′ 1–20 and 5′ 1–20 mut, at 46 °C; polyadenylated 3′ UTR, and the 3′ UTR, at 42 °C; 3′ 1–21, at 46 °C, 3′ 1–47, at 46 °C; 3′ 22–66, at 42 °C; polyadenylated 3′ comp (A) at 60 °C), and 68 °C for 30s, using a Perkin-Elmer Cetus DNA thermocycler. The resulting PCR products were purified by a QIAquick gel extraction G-50 kit (Qiagen) before they were used as templates for RNA synthesis. After transcription, the reaction mixture was treated with DNAse RQ1 (Promega) at 37 °C for 30 min to remove the DNA template in the presence of RNase inhibitors (Promega). Unincorporated nucleotides were removed by precipitation. For the synthesis of radiolabeled and biotin-labeled RNA transcripts, [α-^{32}P]-ATP and [α-^{32}P]-UTP and biotin-16-UTP were included in the transcription reaction, respectively. The synthesis of the human ferritin H-chain ire-responsive element (IRE-fer) region used as the non related RNA was described previously (Solano-González et al., 2007).

Preparation of HeLa and CaCo-2 cell extracts

HeLa and CaCo-2 cell extracts were prepared essentially as described by Barton and Flanegan (1993) with some modifications. Briefly, cell monolayers were washed twice with cold phosphate-buffered saline and once with five volumes of washing buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT). After the final wash, cells were resuspended in two cell volumes of washing buffer. HeLa cells were homogenized with 20 strokes, while CaCo-2 cells were homogenized with 60 strokes in a glass dounce homogenizer. The cell homogenates were clarified by centrifugation at 10,000 rpm for 30 min in a Sorvall GSA rotor; the supernatant was adjusted to 1 mM $CaCl_2$, treated with micrococcal nuclease (15 μg/ml), and incubated at 20 °C for 15 min. The nuclease was inactivated with 2 mM EGTA and the S10 supernatant was centrifuged again at 10,000 rpm for 15 min at 4 °C. The supernatants, called S10 extracts, were aliquoted, and the concentration of proteins in each extract was determined by the Bradford assay (Bradford, 1976). Extracts were stored at −70 °C until use.

UV-crosslinking of RNA–protein complex

UV-crosslinking assays of RNA–protein complexes were performed using a method described previously (Gutierrez-Escolano et al., 2000) in a reaction mixture containing 200 pmol of ^{32}P-labeled RNA and 40 μg of S10 extract. The UV-crosslinked proteins were fractionated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were fixed and dried, and the results were visualized by autoradiography.

5′–3′ co-precipitation assay

The assay was modified from a method described previously (Isken et al., 2003). Briefly, 70 ng (500,000 cpm) of [α-^{32}P]-labeled RNA, 4 μg of biotin-labeled RNA, 10 μg S10 extracts, and 10 μg of tRNA were incubated in a buffer containing 50 mM HEPES, 80 mM $MgCl_2$, 0.1 mM DTT, 120 mM KCl, 0.2% glycerol in a final volume of 20 μl for 15 min at 4 °C. The complexes were incubated with streptavidin-sepharose beads with agitation at 37 °C, for 40 min. The streptavidin-sepharose beads were pelleted by centrifugation and washed twice with buffer C 40 KCl (10 mM HEPES pH 7.6, 0.3 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 5% glycerol, 40 U RNAsin) and once with buffer C 150 KCl. The complexes were treated with proteinase K, and the precipitated RNA was analyzed in a 6% polyacrylamide gel with 7 M urea at 20 mA for 60 min. The gel was dried and visualized by autoradiography. Quantification of the precipitated [α-^{32}P]-labeled RNA under different conditions, was obtained from bands intensities in the scanned images using the ImageJ software (http://rsb.info.nih.gov/ij) and reported as precipitation percentages.

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